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Pea Xyloglucan and Cellulose

V. XYLOGLUCAN-CELLULOSE INTERACTIONS *IN VITRO* AND *IN VIVO*

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ABSTRACT

Since xyloglucan is believed to bind to cellulose microfibrils in the primary cell walls of higher plants and, when isolated from the walls, can also bind to cellulose *in vitro*, the binding mechanism of xyloglucan to cellulose was further investigated using radioiodinated pea xyloglucan. A time course for the binding showed that the radioiodinated xyloglucan continued to be bound for at least 4 hours at 40°C. Binding was inhibited above pH 6. Binding capacity was shown to vary for celluloses of different origin and was directly related to the relative surface area of the microfibrils. The binding of xyloglucan to cellulose was very specific and was not affected by the presence of a 10-fold excess of (1→2)-β-glucan, (1→3)-β-glucan, (1→6)-β-glucan, (1→3, 1→4)-β-glucan, arabinogalactan, or pectin. When xyloglucan (0.1%) was added to a cellulose-forming culture of *Acetobacter xylinum*, cellulose ribbon structure was partially disrupted indicating an association of xyloglucan with cellulose at the time of synthesis. Such a result suggests that the small size of primary wall microfibrils in higher plants may well be due to the binding of xyloglucan to cellulose during synthesis which prevents fasciation of small fibrils into larger bundles. Fluorescent xyloglucan was used to stain pea cell wall ghosts prepared to contain only the native xyloglucan:cellulose network or only cellulose. Ghosts containing only cellulose showed strong fluorescence when prepared before or after elongation; as predicted, the presence of native xyloglucan in the ghosts repressed binding of added fluorescent xyloglucan. Such ghosts, prepared after elongation when the ratio of native xyloglucan:cellulose is substantially reduced, still showed only faint fluorescence, indicating that microfibrils continue to be coated with xyloglucan throughout the growth period.

Plant cell walls exist as a macromolecular complex of various polysaccharides and some proteins (4, 15, 17). When examined by EM, the cross section of primary cell walls often appears multilayered and ordered (22). Changes in wall architecture during elongation or/and expansion could be derived from wall loosening. It has been proposed that xyloglucans function in young plant cell walls as a cementing matrix material which contributes cross-links and rigidity to the cellulose framework (10, 15). The integrity of xyloglucan could then control the ability of microfibrils to loosen and the whole cell to expand during growth. In fact, wall extension is associated with xyloglucan degradation by auxin-induced endoglucanases (11, 14).

The macromolecular complex of xyloglucan and cellulose has been isolated from elongating regions of etiolated pea stems (10). The association between the two polysaccharides is very strong because only concentrated alkali (24% KOH) was effective in dissolving xyloglucan from the macromolecule. Standard reagents for breaking weak hydrogen bonds such as 8 M urea were ineffective, and DMSO only extracted a small part of the xyloglucan. However, the xyloglucan component of the complex was much more accessible to endoglucanase hydrolysis than cellulose microfibrils. There must be a certain ordered mechanism for the assembly and association of two kinds of (1→4)-β-glucans to form such tight complex.

Xyloglucans isolated from plant cell walls can be bound to cellulose *in vitro* (1, 10). Such reconstitution probably creates an organized xyloglucan:cellulose complex. However, the reconstituted complex is not so tightly organized as the native macromolecule because the xyloglucan can be extracted with mild alkali (4% KOH) from the complex (10). Valent and Albersheim (20) studied the association of xyloglucan fragments with cellulose primarily under non-physiological conditions, and little information is available on the nature of the binding of a native xyloglucan to cellulose. The present communication examines the binding of xyloglucan to cellulose using radioiodinated pea xyloglucan. We also show the effect of xyloglucan on the biogenesis of cellulose *in vivo* using the cellulose-producing bacterium *Acetobacter xylinum* (2, 3). *A. xylinum* was employed as a model system for the effect of xyloglucan on cellulose biogenesis *in vivo* because (a) the cellulose is secreted into the medium and the structure of its microfibrils is known to be subject to modification by agents which interact with β-glucan molecules (8, 9) and (b) unlike plant systems, no xyloglucan is produced which could compete with added xyloglucan as was recently demonstrated in studies with pea protoplasts (13). Finally, we have used fluorescent xyloglucan as a probe to study the association of endogenous xyloglucan with cellulose in elongating pea stem cell walls.

MATERIALS AND METHODS

Materials. Fluoresceinamine, cyanogen bromide, laminarin (from *Laminaria digitata*), lichenan (from *Cetraria islandica*), arabinogalactan (from *larch* wood), pectin (from *citrus* fruit), cotton cellulose, carboxymethylcellulose, and xylan (from *larch* wood) were obtained from Sigma. Pustulan (from *Pustulan papullosa*) and pachyman were from Cal-Biochem. Calcofluor was purchased from Ciba-Gigy, Sephadex G-50 from Pharmacia, $Na^{125}I$ (17 Ci/mg) from New England Nuclear, and Iodo-Beads from Pierce.

Xyloglucan Derivatives. To prepare fluorescence-labeled xyloglucan (7), 20 mg of CNBr were dissolved in 0.2 ml of water and added to a solution containing 40 mg of pea xyloglucan in 1 ml of water. The mixture was adjusted to pH 11 and maintained at that pH for 5 min by addition of 0.2 M NaOH. The activated polysaccharide was desalted by gel filtration on a 1 × 20 cm column of Sephadex G-50 in 0.2 M sodium borate at pH 8.0. The polysaccharide fractions (void volume) were pooled and incubated with 4 mg of fluoresceinamine overnight at room temperature. The fluorescein-labeled xyloglucan was separated

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from the unreacted fluoresceinamine by gel filtration on Sephadex G-50 (1 × 20 cm) equilibrated in 50 mM phosphate-buffered saline (pH 7.0). Fractions (1.0 ml) were collected and A at 440 nm was measured. The polysaccharide fractions were measured by the phenol/sulfuric acid method (6), pooled, and stored at −80°C. Calculations showed that 2 μmol of fluorescein were incorporated into the 260 μmol of sugar residues recovered, which corresponds to a degree of substitution of 17 mol of fluorescein per mol of xyloglucan (xyloglucan mol wt = 330,000).

^{125}I-labeled xyloglucan was prepared using Iodo-Beads according to the method of Markwell (16) by reaction of ^{125}I with the fluorescein moiety on xyloglucan. Four Iodo-Beads were washed twice with 50 mM phosphate-buffered saline (pH 7.4) and dried on filter paper. The Iodo-Beads were then added to 200 μl of Na^{125}I solution (2 mCi) and incubated for 5 min. Two mg of fluorescence-labeled xyloglucan (800 μl) were added to the Iodo-Beads solution, and the mixture was incubated for 15 min. ^{125}I-labeled xyloglucan was desalted by gel filtration of Sephadex G-25 equilibrated in 50 mM phosphate-buffered saline (pH 7.4). Specific activity of the polysaccharide was 2.8 × 10^5 dpm/μg xyloglucan.

Polysaccharides. Pea cellulose was prepared (10) from the elongating region (1 cm) of the third internode of pea epicotyls (7 d). The tissues were ground with liquid N_2 and extracted 6 times with 24% KOH/0.1% $NaBH_4$. The insoluble materials were neutralized with acetic acid, dialyzed against distilled H_2O, and freeze dried.

The culture of *Acetobacter xylinum* was a generous gift from Dr. M. Benziman, the Hebrew University, Jerusalem, Israel. Pellicles were produced at liquid/air interfaces of standing cultures in Schramm and Hestrin's (19) glucose medium at 25°C. Pellicles, harvested after 48 h, were washed and ground with liquid N_2 in a mortar, and the powder was extracted with 4% KOH/0.1% $NaBH_4$ for 4 h. The mixture was centrifuged and washed 4 times with 4% KOH. The insoluble cellulose was suspended in 2 ml of 1 N acetic acid, dialyzed against distilled H_2O, and freeze dried.

Valonia ventricosa cellulose was kindly provided by Dr. A. D. French. The cellulose had been cleaned and scoured in 2% NaOH and 0.5% PreChem 70 under argon. It had been rinsed in boiling water twice, neutralized in 1% acetic acid, and rinsed in cold water 3 times. All celluloses were further ground with liquid N_2 and the resulting fine powder was suspended in water at 2 mg/ml.

Pustulan was dissolved in 1 N NaOH/0.1% $NaBH_4$ and the mixture was incubated at room temperature for 2 d. The mixture was then neutralized with acetic acid, dialyzed against distilled H_2O, and freeze dried. The deacetylated pustulan was confirmed as a (1→6)-β-glucan by methylation analysis.

Lichenan was dissolved in hot water and purified by dissolution freezing and thawing (21). This procedure was repeated three times.

Assay for Binding of Xyloglucan to Cellulose. Standard reaction mixtures contained 30 μg of [^{125}I]xyloglucan (6.3 × 10^5 dpm), 100 μg of pea cellulose, 25 mM sodium acetate (pH 5.0), and 0.01% NaN_3 in the total volume of 400 μl. The mixture was shaken at 40°C for 6 h. The cellulose was collected and washed by centrifugation 3 times with 25 mM sodium acetate (pH 5.0). A sample of the insoluble cellulose was counted using a Beckman 5500 Gamma counter.

For competition tests with various polysaccharides, 100 μg of polysaccharide were added to 5 μg of [^{125}I]xyloglucan (2.6 × 10^5 dpm) and 100 μg of pea cellulose suspended in 25 mM acetate buffer (pH 5.0). At equilibrium, approximately 5 μg of xyloglucan bind to 100 μg of pea cellulose in the standard mixture.

Microscopy. In order to stain cellulose microfibrils with fluorescent xyloglucan, 50 μl of fluorescence-labeled xyloglucan solution (20 mg/ml) were incubated with 1 ml of pea cell wall ghosts (2 mg carbohydrate as glucose equivalents) and 50 μl of 100 mM sodium acetate (pH 5.0) containing 0.01% NaN_3 in the dark at 40°C with shaking. After 6 h, the ghosts were washed twice with 5 mM sodium acetate (pH 5.0) and once with 5 mM phosphate buffer (pH 6.8), and mounted on slides for fluorescence microscopy. Samples were observed with Zeiss Photomicroscope III equipped with an XBO 75 W/2 Xenon light source using a 450 to 490 nm excitation filter and a 520 nm barrier filter.

Cellulose microfibrils were prepared for EM by staining on carbon-coated copper grids with 2% (w/v) aqueous uranyl acetate, using bacitracin as a spreading agent (2). Grids were examined using a JEOL 100 CX II electron microscope and images were recorded on Eastman Kodak 4489 Estar thick base film.

RESULTS

Kinetics and Properties of the Binding of Xyloglucan to Cellulose. Binding of [^{125}I]xyloglucan to pea cellulose proceeded and reached equilibrium within 4 h at 40°C showing saturation binding of about 5 μg of xyloglucan per 100 μg of pea cellulose (Fig. 1). The binding of xyloglucan to cellulose was examined as a function of pH (Fig. 2). The binding occurs at pH values less than pH 6, and inhibition of binding was observed in alkali; 50% inhibition was observed at pH 7. High salt solution also inhibited the binding; 1 M NaCl inhibited binding by 50% (data not shown).

Various polysaccharides were examined for their ability to inhibit the binding of [^{125}I]xyloglucan to cellulose (Table I). The polysaccharides were added in the reaction mixture at 10-fold higher concentration than that of [^{125}I]xyloglucan. β-Glucans containing 1→2 linkages (1→2-β-glucan from *Agrobacterium radiobacter*), 1→3-linkages (laminarin and pachyman), 1→6-linkages (pustulan), or 1→3-and 1→4-β-linkages (lichenan) did not inhibit the binding. However, 50% inhibition was obtained by the addition of xylan, probably because the xylan consists of (1→4)-β-linked xylopyranosyl residues similar to (1→4)-β-glucan structure. These results indicate that the binding occurs specifically with structures containing (1→4)-β-glycosyl linkages in polymers having a complementary conformation to cellulose. Plant matrix polysaccharides such as arabinogalactan and pectin did not show any inhibition of binding. Therefore, of various primary wall polysaccharides tested, the binding of xyloglucan appears to be specific to cellulose.

Cellulose microfibrils from different sources were used as

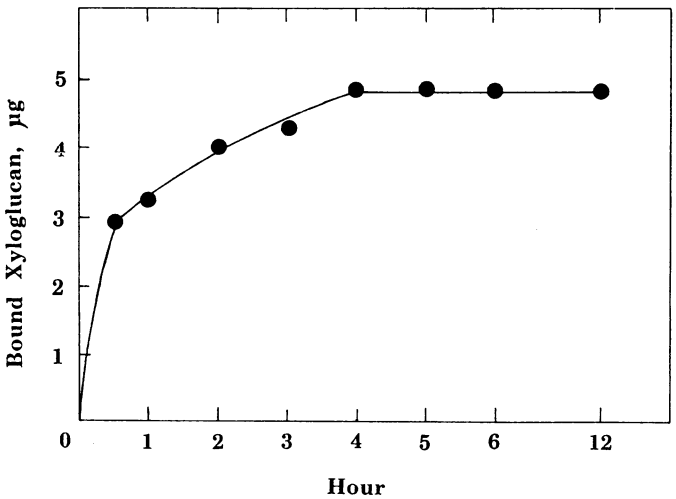

FIG. 1. Time course of binding to cellulose of [^{125}I]xyloglucan. Reactions contained 100 μg pea cellulose and were incubated at 40°C.

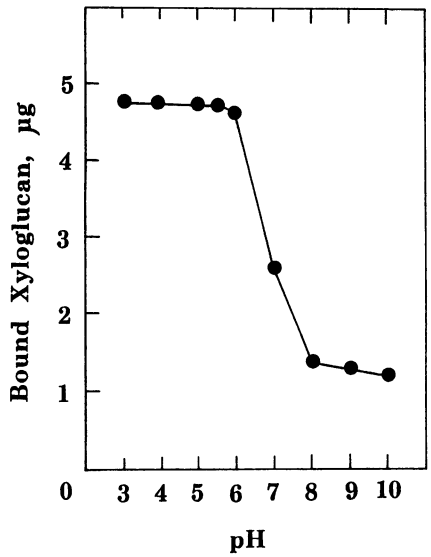

FIG. 2. Effect of pH on the binding to cellulose of [^{125}I]xyloglucan. Several pH buffers were used at 25 mM concentrations: sodium citrate (pH 3 and 4); sodium acetate (pH 5.0 and 5.5); potassium phosphate (pH 6 and 7); sodium borate (pH 8, 9 and 10).

Table I. *Relative Binding Activity of [^{125}I]Xyloglucan to Pea Cellulose in the Presence of Various Polysaccharides*

Each reaction contained 5 µg [^{125}I]xyloglucan, 100 µg of cellulose.

Polysaccharide	Relative Binding Activity
	%
None	100
(1→2)-β-Glucan	103.4
Laminarin	99.3
Pachyman	100.7
Pustulan	99.2
Lichenan	92.0
Xylan	47.0
Arabinogalactan	106.7
Pectin	107.9

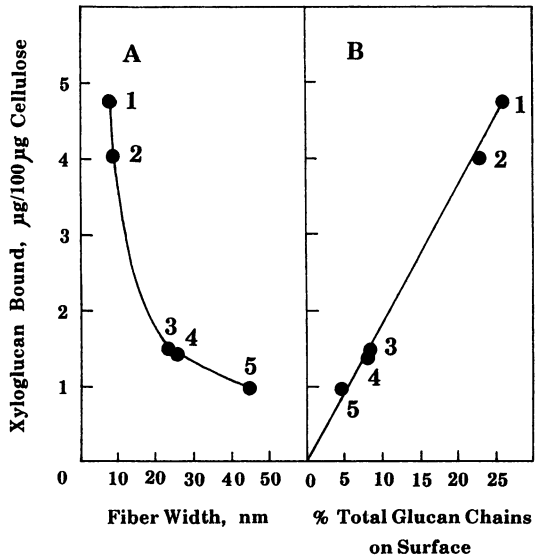

FIG. 3. Binding capacity of [^{125}I]xyloglucan to various celluloses. 1, pea cellulose; 2, cellulose from *A. xylinum* grown in Calcofluor; 3, *Valonia* cellulose; 4, cotton fiber cellulose; 5, *A. xylinum* cellulose.

substrates to examine the binding capacity (Fig. 3). The average width of each cellulose microfibril was estimated by measurement of fiber widths in negatively stained preparations using EM. The average fiber widths of *Valonia*, cotton, and *Acetobacter* celluloses were measured as 24 nm, 26 nm, and 45 nm, respectively. *A. xylinum* was also cultivated with culture medium containing 0.5% Calcofluor, and fine fibers (about 9 nm) were obtained. These fibril widths may not be a true reflection of diameters in the native celluloses, but are what were observed in the samples following the preparation procedures described in "Materials and Methods." The binding capacity of [^{125}I]xyloglucan to cellulose was dependent on the fiber diameter (Fig. 3A). On the assumption that a 3.2 nm fibril contained 36 chains (5), we estimated the percent of fibrils on the surface of each of these celluloses; when binding is plotted as a function of surface fibrils (Fig. 3B), binding is shown to be a linear function of surface available.

Effect of Xyloglucan on the Viscosity of Carboxymethylcellulose. Interaction between carboxymethylcellulose and pea xyloglucan was observed viscometrically. The relative viscosity of pea xyloglucan was barely detectable even at 1.0% (w/v). The viscosity of carboxymethylcellulose solutions (1.0%) was increased by the addition of pea xyloglucan (Fig. 4), indicating that higher mol wt complexes of soluble cellulose with xyloglucan were created.

Effect of Xyloglucan on the Biogenesis of *Acetobacter* Cellulose. To examine the effect of xyloglucan on cellulose biogenesis *in vivo*, pea xyloglucan was added to a cellulose-forming culture of *A. xylinum*. After 2 d cultivation, samples were washed with 4% KOH in order to remove xyloglucan and proteins, and microfibrils were observed after negative staining for EM. *A. xylinum* produces crystalline 1.5 nm fibrils in association with intracellular synthesizing sites (8). The microfibrils bind together into an extracellular ribbon of cellulose (Fig. 5A). In the presence of pea xyloglucan (0.1%), microfibrils of relatively small diameters were observed indicating disruption of ribbon assembly (Fig. 5B). Pea cellulose was also observed (Fig. 5C) by electron microscopy. The fibrils still retained ordered orientation, and the microfibril width was very small (8 nm).

Visualization of Pea Cellulose Network using Fluorescent Xyloglucan. Pea cell wall ghosts containing only the native xyloglucan:cellulose network or only cellulose could be visualized by fluorescence microscopy after staining 4% KOH-insoluble and

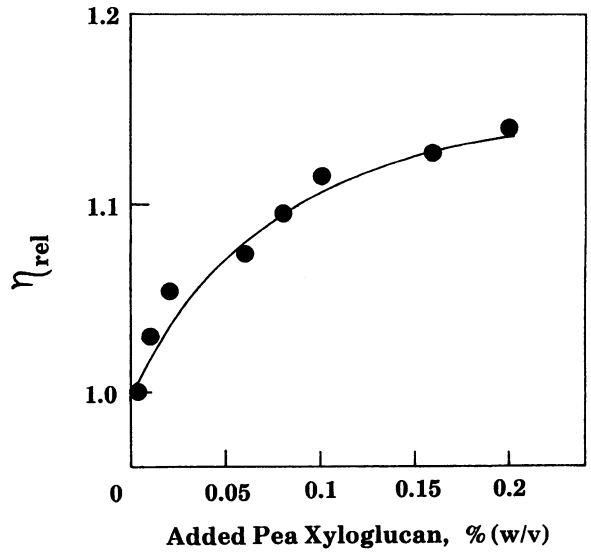

FIG. 4. Effect of xyloglucan on the viscosity of a 1% (w/v) carboxymethylcellulose solution.

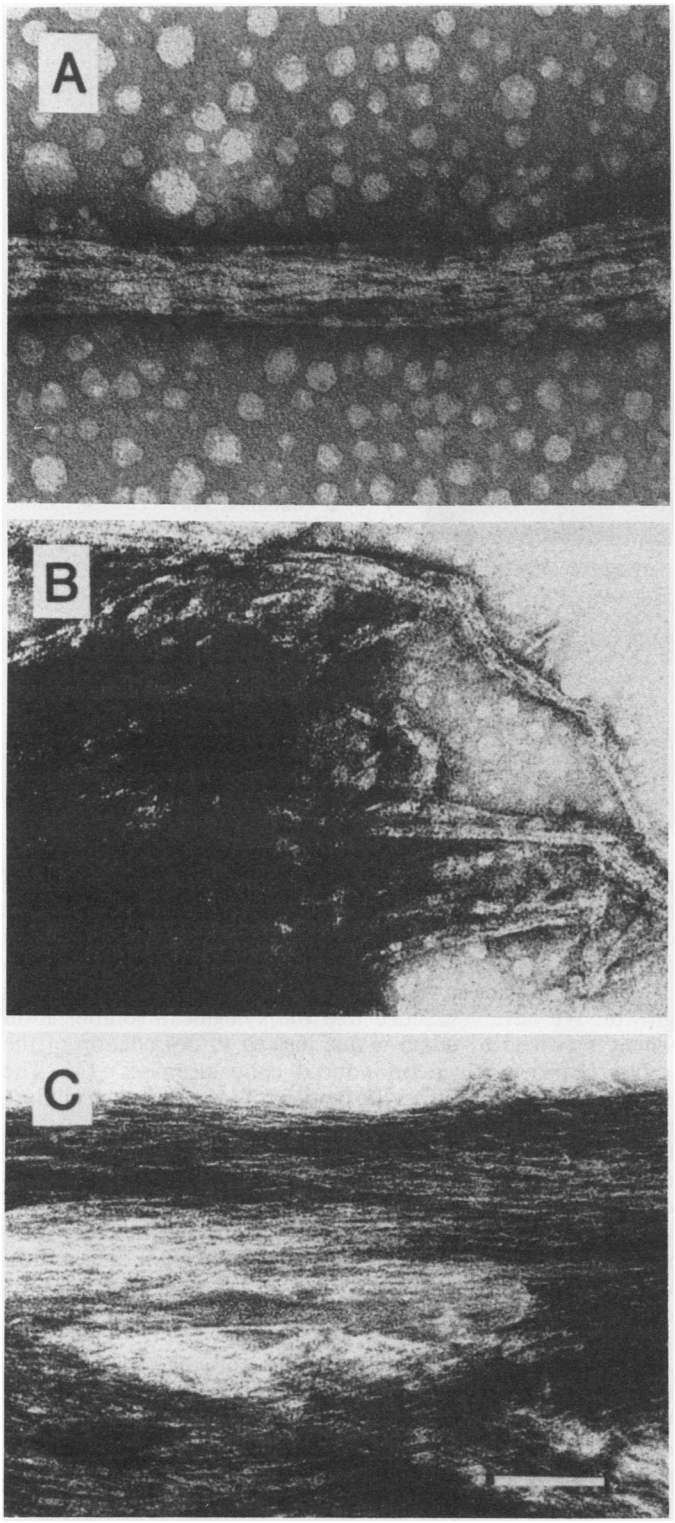

FIG. 5. Negatively stained cellulose fibers. A, Microfibrillar ribbon produced by *A. xylinum*; B, *Acetobacter* cellulose fibers formed in the presence of pea xyloglucan; C, cellulose fibers of young pea epicotyl cells. Magnification bar is 100 nm.

24% KOH-insoluble cell wall residues, respectively, with fluorescence-labeled xyloglucan. Cell wall ghosts containing only cellulose still showed recognizable cell shapes with fluorescence distributed over the entire wall surface when xyloglucan was removed from the material with 24% KOH (Fig. 6A). The fluorescence was most intense in bands that traversed the ghosts in a vertical direction in relation to the cell axis. During growth, cellulose and xyloglucan levels increase differently. Xyloglucan levels per segment double after 48 h, and cellulose levels increase 12-fold (14), and thus the final cellulose level per cell is higher in elongated (9.0 mg/g fresh weight) and auxin-treated (14 mg/g fresh weight) cells than in cells about to grow (zero time, 3.3 mg/g fresh weight) (Fig. 6, A–C). Therefore, one might predict that, after elongation or expansion (auxin-treated), ghosts containing the xyloglucan:cellulose network might have more available sites for binding of added fluorescent xyloglucan. The fact that such ghosts still display only weak fluorescence (Fig. 6, D–F) was surprising and indicated that the microfibrils are extensively covered with xyloglucan even when the cellulose:xyloglucan weight ratio is substantially increased from 1.4 in the initial small cells to 8.7 in elongated cells and to 4.5 in auxin-treated cells after 48 h growth (8).

DISCUSSION

We have directly demonstrated the binding of pea xyloglucan to cellulose *in vitro* (Figs. 1–3). The results obtained strongly support the notion that xyloglucan is bound to the surface of cellulose microfibrils in the primary cell wall of higher plants. Since other glucans do not inhibit the binding of xyloglucan to cellulose (Table I), the association between the two kinds of (1→4)-β-glucans occurs specifically. Since xyloglucans exhibit no self-affinity (12), cellulose microfibrils appear to be covered with a monolayer of xyloglucan in a reconstituted complex. The finding that (1→4)-β-xylan shows some competition with xyloglucan for binding raises the interesting possibility that the heteroxylans found in many monocot walls may also interact with cellulose, although such walls also contain some xyloglucan presumably bound to cellulose (17). We have not, however, examined directly any interaction between xylans and cellulose or xyloglucan.

Based on the binding capacity for cellulose microfibrils of differing surface area, the capacity was dependent on the surface area of the microfibrils (Fig. 3). However, the native xyloglucan:cellulose complex contains 14-fold higher levels of xyloglucan than those in the reconstituted complex (10). In the experiments presented here, 100 μg of pea cellulose were saturated with 5 μg of xyloglucan. If 25% of the fibrils of the pea cellulose used in these studies are exposed to the surface and the xyloglucan contained about 60% by weight (1→4)-β-glucan equivalents, then it would appear that only about 10 to 15% of the available surface area was covered at equilibrium, under the conditions of our experiment. In the primary cell walls of pea stems, xyloglucan probably not only binds to the surface of cellulose microfibrils but also weaves into the amorphous parts of microfibrils. This probably explains why concentrated alkali, which causes microfibrils swelling, is required for the extraction of xyloglucan (4, 10); mild alkali which does not cause microfibril swelling does not dissociate the complex even though it does prevent new associations of xyloglucan and cellulose (Fig. 2). Thus, the native association may form close to or directly at the site of cellulose synthesis and this could enhance binding *in vivo*. This hypothesis is supported by the recent observation that exogenous xyloglucan does not complex well with the newly formed cellulose microfibrils during the regeneration of cell walls of pea protoplasts (13).

It has been shown that Calcofluor and carboxymethylcellulose alter the assembly of cellulose microfibrils by *A. xylinum* (8, 9). An increase in the rate of polymerization was accompanied by an alteration of ribbon assembly when carboxymethylcellulose was added to cellulose-synthesizing cultures of *A. xylinum*. Haigler and Benziman (8) showed that carboxymethylcellulose interfered with ribbon assembly at a higher level than does Calcofluor. Carboxymethylcellulose associates with the subunits of the ribbon and prevents the fasciation into larger bundles, whereas Calcofluor acts at an earlier stage of assembly preventing associ-

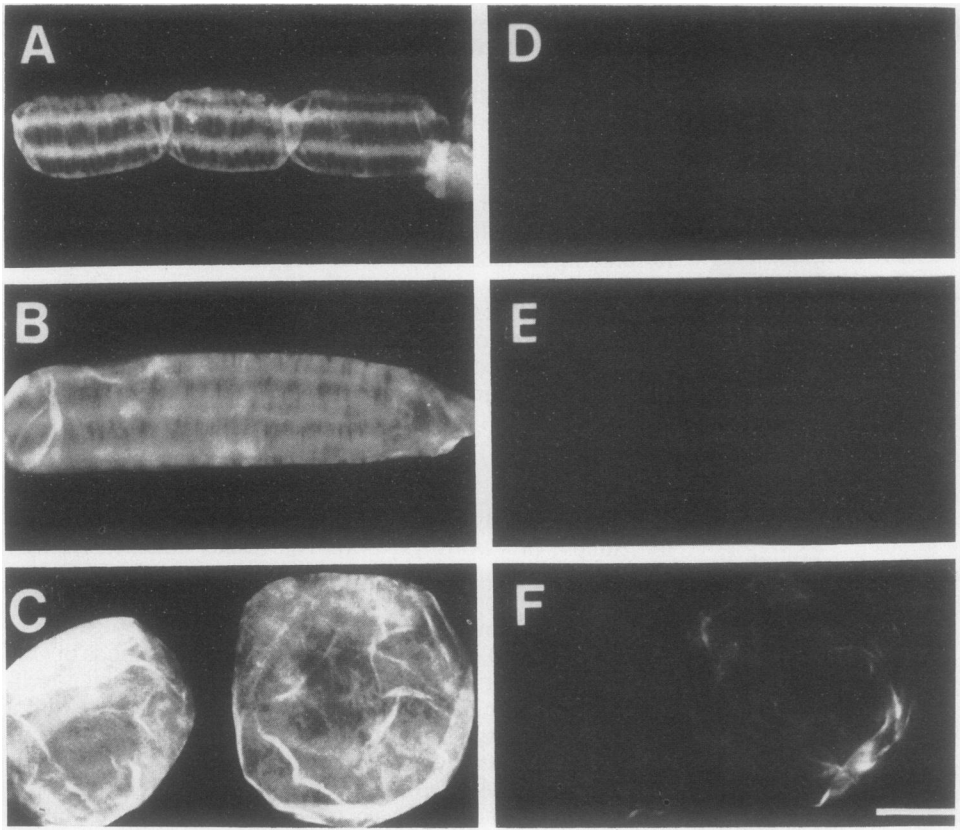

FIG. 6. Fluorescence microscopy of cell wall ghosts stained with fluorescence-labeled xyloglucan. The ghosts were prepared from the elongating region of pea epicotyls at 0 time (A and D) and at 2 d from untreated (B and E) and auxin treated (C and F) tissue. The ghosts D to F are composed of xyloglucan and cellulose and A to C contained only cellulose before addition of fluorescent xyloglucan. Magnification bar is 20 μm.

ation of the 1.5 nm subelementary fibrils into larger microfibrils. Our results show that xyloglucan also probably interferes with ribbon assembly at a level similar to that caused by carboxymethylcellulose (Fig. 5, A and B). In the primary cell walls of pea stems, therefore, the microfibril size may be controlled by xyloglucan. The surface area of microfibrils in pea stems is extensively covered with xyloglucan during cellulose deposition and, in fact, the diameters of microfibrils are very small in the primary cell walls of pea stems (Fig. 5C) and may represent the fibril size generated by a single cellulose synthase complex. Xyloglucans probably function in the organization of microfibril assembly to prevent the fasciation of such fibrils and to spread the fiber network over the cell surface (Fig. 6). In addition, this may stimulate the rate of cellulose synthesis, as has been observed for cellulose synthesis in *A. xylinum* in the presence of Calcofluor and carboxymethylcellulose (8).

A decrease in xyloglucan deposition is accompanied by an increase in cellulose deposition during elongation of pea stems (14). Although the cellulose content increases 12-fold in cell walls after 2 d of elongation, xyloglucan increases only about 2-fold. Nevertheless, cellulose fibers continue to be extensively coated with xyloglucan during cellulose deposition (Fig. 6). The original xyloglucan may be partially degraded during elongation and possibly reconstituted with newly-formed fibers to create an altered microfibril network.

Acidic pH stimulates cell-wall loosening (18), and auxin-induced cell growth is accompanied by a decrease in the pH of the medium. Also, treatment of pea tissue with auxin leads to solubilization of part of the insoluble xyloglucan (14). It was proposed that cell wall extension occurs by a hydrogen bond creep between xyloglucan and cellulose fibers (15). Valent and Albersheim (20), using fragments of xyloglucan, concluded that binding to cellulose was unaffected by pH in the range of 1.9 to 6.7. The result presented in this paper, using native and high mol wt xyloglucan, shows that the interconnection between the two polymers is stable at acidic pH (less than 6) and unstable above this pH (Fig. 2). These results further confirm that the creep between the two kinds of β-glucans does not occur at acidic pH, and therefore, it is highly unlikely that xyloglucan chains creep along the surface of the cellulose fibers during acid-induced growth. We have concluded that the xyloglucan solubilization which is evoked by auxin is due instead to degradation of the polysaccharide with auxin-induced endoglucanases (14). The effect of high pH or salt on the binding of xyloglucan to cellulose is consistent with the notion that these molecules interact by hydrogen bonding. However, it is somewhat surprising that the interaction is inhibited even under relatively mild alkaline conditions. The pH dependence of binding must reflect a real change in the affinity of the two types of polymer for each other since the labeled xyloglucan used here has no charged groups and is, in itself, stable from pH 3 to 10. It would thus seem that the details of the precise chemical interaction between these polymers merit further study.

Xyloglucans can be a useful tool for studying cellulose structure and synthesis because the polysaccharides resemble an antibody for cellulose. Visualization of cellulose could contribute to our understanding of the morphology of plant cell walls, and to the localization of cellulose (Fig. 6). [^{125}I]Xyloglucan could be used as a means of determining the exposed surface areas of various types of cellulose (Fig. 3), and may be useful for detecting very low levels of cellulose. For studies on the biosynthesis of cellulose *in vitro*, one might specifically detect cellulose produced by binding of radioactive xyloglucan, thus eliminating the need for a radioactive substrate or for subsequent tedious structural analyses. The interaction of xyloglucan with carboxymethylcellulose (Fig. 4), indicates that xyloglucans might also be used as viscosity modifiers for various soluble β-glucan or β-xylan derivatives.

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